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Asymmetric organotellurides as potent antioxidants and building blocks of protein conjugates

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New asymmetric organotellurides exhibiting good antioxidant properties *in vitro* and in cell culture can be attached to human serum albumin.

Introduction

Numerous human diseases, ranging from Rheumatoid Arthritis to neurodegenerative diseases, are associated with an imbalance in cellular redox systems, a biochemical condition known as Oxidative Stress (OS).[1–3] The use of either natural, or synthetic antioxidants is therefore increasingly considered in Medicine to target OS related disorders. The broad spectrum of antioxidants divides into different classes, among which catalytic antioxidants, such as enzymes and enzyme mimics, play an important role.[4] Unlike 'one shot' antioxidants, such catalysts are able to counteract OS at low concentrations, and are also sensitive towards the presence of their stressor (and reducing) substrates, hence endowing them with a certain degree of selectivity.[5,6]

Catalysts mimicking the activity of the human enzyme glutathione peroxidase (GPx) are particularly interesting,[7] and one of them, 2-phenyl-1,2-benzoisoselenazol-3(2*H*)-one (ebselen), is currently being evaluated as therapeutic antioxidant in a clinical trial.[8] The practical use of such antioxidants is, however, often limited, due to—among others—low catalytic activity, low solubility in aqueous media, lack of selectivity and an unfavourable pharmacokinetic transport and distribution profile in the body.

In theory, many of the more biochemical problems associated with the use of synthetic GPx mimics might be avoided if such highly active catalysts were integrated into human transport proteins, such as human serum albumin (HSA). If successful, the resulting mimic–protein conjugates would then exhibit the activity of one protein (*i.e.* GPx), but travel along the path of another (*i.e.* HSA), possibly even without causing an immunogenic reaction.

A similar, yet chemically much simpler approach has already successfully been applied to deliver nitric oxide (NO) to cells, *i.e.* by *S*-nitrosylating cysteine residues in the protein.[9] Recent attempts to endow enzymes with antioxidant activity, either by genetic or chemical modification, have been met with mixed success.[10–12] Here we show that it is, at least in principle, also possible to 'load' highly active catalysts onto HSA. It should be pointed out from the beginning that results presented here are designed to demonstrate the feasibility of the first steps of this approach for larger molecules, such as GPx mimics. This paper does not intend to provide a full investigation of the biochemical and pharmacological properties of the resulting antioxidants and antioxidant conjugates, nor does it promote any of the compounds as potential drugs.

Results and discussion

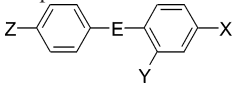
In order to construct a catalyst–protein conjugate, it is first necessary to define catalyst molecules suitable for protein attachment. For this purpose, asymmetric tellurides **1** to **3** were chosen (see Table 1), since the presence of a single amino group allows covalent attachment to lysine residues of proteins without risk of cross-linking.[13] In addition, amino, as well as methoxy groups are known to increase catalytic activity, and compounds such as **2** and **3** might therefore be expected to be highly active and readily attachable to transport proteins.[14]

Asymmetric chalcogen compounds **2–4** were not yet known in the literature. They were therefore synthesised successfully following the method of Suzuki *et al.* that has previously been employed to synthesise **1**.[15] This synthetic method is comparably straightforward, and likely to be also suitable for a range of other asymmetric catalysts. In the context of this study, it was used to endow the catalyst with two important substituents on the aromatic rings, *i.e.* a methoxy group for increased activity, and an amino group for protein attachment.

The compounds were then evaluated in two assays indicative of catalytic activity, the thiophenol (PhSH) assay for GPx activity[16] and in the metallothionein (MT) assay for peroxidation catalysis.[17–19] In line with the above considerations for increased activity, **2** and, to a lesser extent, **1** and **3**, were highly active in both assays, by far exceeding the activity of ebselen (Table 2). While 100 μM ebselen led to a rate of PhSSPh formation of 0.89 μM min^{-1} in the PhSH assay, the same amount of **2** was more than 56 times as active (50.5 μM min^{-1}). Telluride **1** showed a similar increase in efficiency (51 times as active as ebselen), while **3** was slightly less active, but still 25 times as active as ebselen.

In order to confirm this activity in another, independent assay, and to avoid the use of methanol as solvent, the compounds were also tested in the well-established metallothionein assay

Table 1 Structure of compounds used in this study



Cmpd	X	Y	Z	E
1	NH_2	H	H	Te
2	NH_2	H	OMe	Te
3	H	NH_2	OMe	Te
4	H	NH_2	OMe	Se

† These authors contributed equally.

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Table 2 Electrochemical and *in vitro* results for compounds **1** to **4**. Experimental details are given in the text. Second order rate constants k are given separately. IC_{50} values refer to 50% zinc release from MT in the MT oxidation assay

Cmpd	Epa_1/mV (±10 mV)	Epa_2/mV (±10 mV)	ΔE_1/pH	ΔE_2/pH	Initial reduction rate of $H_2O_2/10^{-7}$ M s^{-1} ±5%[a]	k(PhSH)/M^{-1} s^{-1}	MT% Zn Release ±5%[b]	Initial rate of Zn release/ 10^{-10} M s^{-1} ± 5%[c]	k(MT)/M^{-1} s^{-1}	IC_{50}/nM[c]
1	+411	+894	−42	−46	7.63	3.5	75	9.3	2.66	35
2	+388	+860	−50	−42	8.42	4.0	98	30.6	8.74	25
3	+374	+800	−67	−42	3.82	1.5	88	20.8	5.94	35
4	+700[d]	—	nd	na	nd	na	na	na	na	>1000
Ebselen	+1044	—	nd	na	0.15	0.05	23.5	0.75	0.21	3320

[a] Catalysts used in assays at 100 µM. [b] 100 nM. [c] 200 nM. [d] Only one peak observed. na: not applicable, nd: not determined.

(MT assay, see Experimental section). As for the PhSH assay, **2** was also highly active in the MT assay, where it facilitated almost complete (98%) oxidation of MT by the peroxide within 60 min (Table 2). **3** and **1** were only slightly less active, with 88% and 75% release, respectively, while ebselen was dramatically less active with just 23.5% release within 60 min. Together, the PhSH and MT oxidation assays confirm high peroxidation activity of **2** and **3** under independent experimental conditions.

Interestingly, the activity of the agents in both assays was comparable, with second order rate constants for H_2O_2 reduction (in the PhSH assay) and zinc release (in the MT assay) within the same order of magnitude (Table 2). This is perhaps not too surprising, considering that the underlying catalytic mechanism in both assays involves initial peroxidation of the catalyst to telluroxide, *i.e.* both reactions might share similar rate determining steps.[14]

It should be pointed out that the activities of **2** and **3** in the MT assay are among the highest observed for diaryl tellurides to date,[14] and are in sharp contrast to the almost complete lack of activity for ebselen in this particular assay. Although it was not the prime target of this study to increase activity of organochalcogens, the presence of the amino group clearly had a beneficial effect on the activity of the compounds, at least in the MT assay.

The activity of these compounds was then further evaluated in two different studies, one aiming at a better understanding of the redox behaviour of the compounds, the other evaluating their activity in living cells. Cyclic Voltammetry (CV) was used to gain insight into the underlying redox chemistry, while cell culture using SK-MES-1 cells was used to estimate the antioxidant behaviour on intact cells.

Using CV, we have recently shown a reasonable correlation between electron-rich substituents on the aromatic ring, the first oxidation potential (Epa_1) and catalytic activity of chalcogen compounds.[14,20] This relationship was supported by **1–4**: As might be expected, all tellurides had two irreversible oxidation peaks, one typical for the one electron tellurium oxidation around 400 mV *vs.* SSE (Epa_1) and one for aniline oxidation between 800 and 900 mV (Epa_2) (Fig. 1, Table 2). The influence of the methoxy group on the Epa_1 values was clearly apparent, and the lowest Epa_1 value was observed for **3** (378 mV), while 'methoxy-free' **1** had an Epa_1 value of 411 mV.

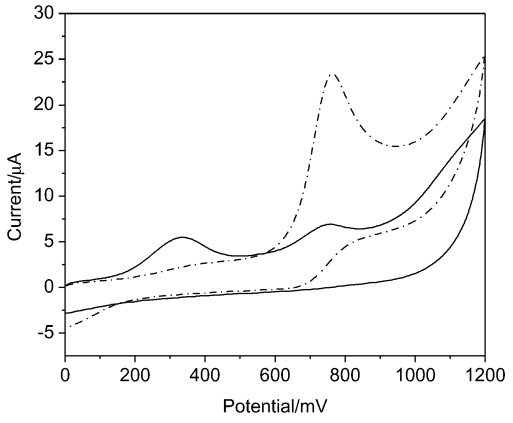

Fig. 1 Cyclic voltammogram of **3** (solid line), and aniline (broken line) for comparison. Compounds were scanned at 200 mV s^{-1} with a glassy carbon electrode and SSE in potassium phosphate buffer (50 mM containing 30% MeOH, pH 7.4) at room temperature.

In line with previous results, the selenide **4**, used here mostly as a control, had a considerably higher first oxidation potential (700 mV), likely to be due to aniline oxidation, with no oxidation peak for selenium oxidation observed in the potential range under investigation.[14,20] This compound was therefore of less interest for further studies.

Interestingly, CV also confirmed that tellurium oxidation in **1–3** (Epa$_1$) is under pH control (Table 2). Under experimental conditions, a pH increase from 5 to 8 resulted in a sharp decrease of Epa$_1$ from around 400 mV to around 250 mV (ΔEpa$_1$/pH between −42 mV and −67 mV). In contrast, the aniline oxidation potential of the three tellurides, which is also under pH control, showed a rather steady decrease from around 1100 mV to around 800 mV between pH 3 and 9 (ΔEpa$_2$/pH between −42 mV and −46 mV). In both cases, sensitivity to pH changes is likely to be due to protonation of the aniline moiety, which itself provides a redox centre at higher electrochemical potentials (Epa$_2$). As a consequence, **1** to **3** incorporate two interdependent electron transfer sites, both of which might be relevant for biological activity, and both of which are under pH control (Fig. 1).

In the context of this study, the focus was on the tellurium redox centre, which was oxidised at considerably lower electrochemical potentials compared to the aniline. Nevertheless, future studies might well investigate further the complex interplay between the two redox centres in 'chemically simple' molecules such as **2** and **3**.

Although the combination of the electrochemical and *in vitro* assays enables the estimation and rationalisation of the catalytic properties of the compounds, the ultimate selection tool for antioxidant activity short of animal testing remains the cell culture assay.

Antioxidant properties of the most promising candidates, *i.e.* **2** and **3**, were therefore tested in a lung carcinoma cell line (SK-MES-1), which is naturally under OS.[21] In spite of being a cancer cell line, SK-MES-1 provides a good model to study the activity of redox active compounds as it negates the need, but still provides the opportunity, to apply 'external' stressors such as H_2O_2. Fig. 2 shows the results obtained in these cell culture studies. It should be mentioned from the outset that these results are preliminary, and considerably more detailed cell culture studies are needed to fully evaluate the protective effects of **2** and **3**, and their precise mode of action in SK-MES-1 and other cell cultures.

In the first set of experiments, **3** (or ebselen) were added to cultured SK-MES-1 cells under internal oxidative stress, *i.e.* in the absence of external stressors. As anticipated for an antioxidant, addition of **3** to cultured cells reduced this internal oxidative stress and significantly increased cell survival by up to 38.5% (at 50 nM) as measured by the rate of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) dye reduction (Fig. 2a). This protective effect resulted in survival rates above the 100% observed for the cells under internal stress, and was statistically relevant ($P < 0.01$ for most data points). It was observed at low nanomolar (5–100 nM) concentrations, indicating high efficiency of the compound in low doses. Importantly, **3** was considerably more active than ebselen, which showed hardly any statistically significant changes in the cell line used (Fig. 2b).

In order to investigate the antioxidant properties of **2** and **3** further, cells were challenged with external oxidative stress, *i.e.* a bolus addition of 50 μM H_2O_2. The latter resulted in severe OS and a reduction of cell survival to approximately 59%. **3**, at just 25 nM, was able to (statistically) significantly ($P < 0.01$) protect cells against H_2O_2, with cell survival increased to 76%. In contrast, ebselen (1 nM–10 μM) could not prevent the damage inflicted by the bolus addition of H_2O_2.

It should be noted that neither **3** nor ebselen were able to fully restore cell survival to 100%. This is perhaps not surprising, considering the order of magnitude difference between catalyst and peroxide concentrations. In addition, the catalyst requires reducing substrates, such as glutathione, and is in competition with damaging peroxidation reactions, such as peroxidation of redox sensitive cysteine proteins, that occur at the same time. An increase to 76% can therefore be seen as a reasonably good antioxidant protection, and is far better than the one observed for the ebselen benchmark.

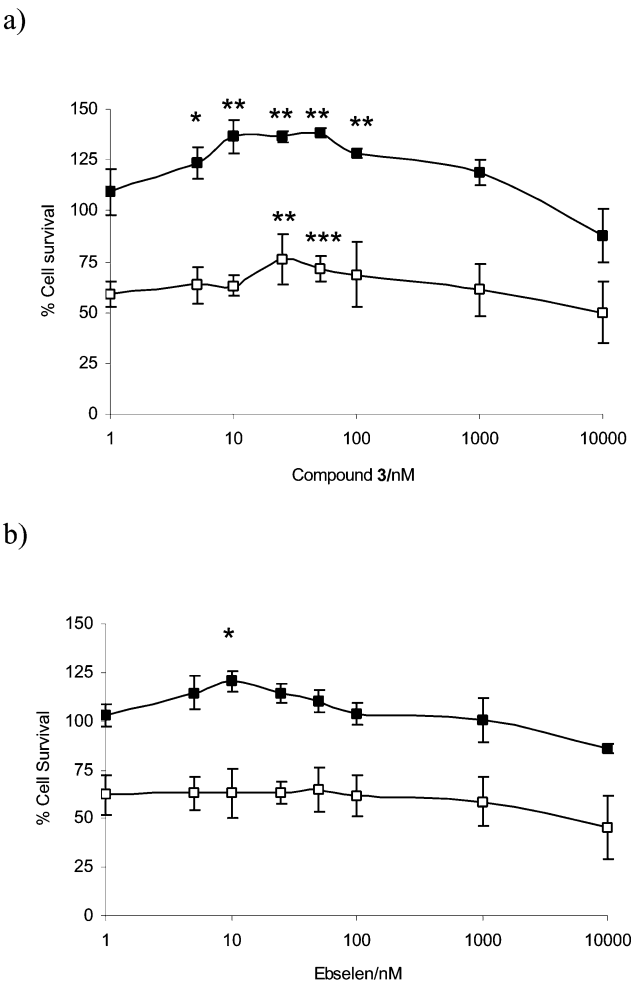

Fig. 2 Cell survival rate of SK-MES-1 cells exposed to (a) compound **3** or (b) ebselen, pretreated with 50 μM H_2O_2 (□) or buffer (■). Data are based on untreated cells as 100% viable. Cells treated with H_2O_2 gave 59% cell viability. Values are means ±SD of four replicates. Student's t-test: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ relative to respective controls. Experiments were reproduced on three separate occasions.

Overall, the cell culture results suggest that **3** has some antioxidant properties in SK-MES-1 cell culture at low, nanomolar concentrations. It should be mentioned that it is unlikely that stimulation of mitogenesis may have accounted for enhanced cell survival, as the assay length was less than half of the cells' doubling time and microscopic observation showed no evidence for this response. Furthermore, the effect was prevalent under H_2O_2-induced stress conditions.

Surprisingly, **2**, the most active of the tellurides *in vitro*, showed only a slight activity in cell culture that was not statistically significant. As is often the case, *in vitro* assays only model a certain number of *in vivo* properties, and the dramatic difference in SK-MES-1 cell culture activity between **2** and **3** might be due to factors not apparent in the GPx or MT assays. Then again, other cell lines might provide a different compound ranking.

Apart from emphasising the importance of using cell culture screens in addition to *in vitro* assays and electrochemistry, the studies with cultured SK-MES-1 cells finally enable the selection of **3** as the most promising candidate for the catalyst–protein conjugate. As for the protein, HSA is an obvious choice, due to its transport proteins as a plasma protein and its previous use as NO carrier. In addition, the lysine residues of albumin are frequently used to anchor signalling molecules, such as in (commercially available) fluorescein-labelled albumin.

There are several methods to link a molecule bearing a NH_2 group to a protein, although most of them have been used

for the coupling of primary amines, not anilines. In the end, the thiophosgene method,[13] that was recently used by Werts *et al.* to couple a fluoresceinamine derivative to avidin, was also successfully employed here to convert the amino group in **3** into an isothiocyanate moiety that was then able to attach to the lysine residues of HSA (Fig. 3).

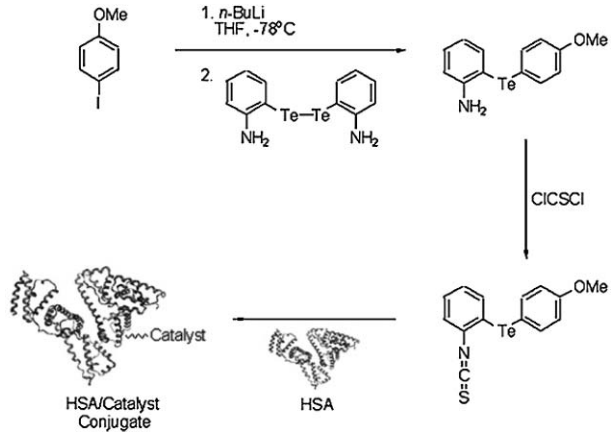

Fig. 3 Scheme of synthetic pathway for HSA–catalyst conjugate.

This method proved far superior to the other coupling methods tried[22] and led to an approximate average labelling ratio of 0.5 catalyst molecules per HSA molecule, as estimated by UV/VIS spectrometry (at 322 nm for label concentration) and Bradford assay (for HSA concentration). Since HSA contains 16 lysine residues, this only represents an average ratio and multi-labelled HSA with higher catalytic activity cannot be ruled out at this stage. Future studies might consider more specific labelling techniques, possibly aiming at less abundant amino acid side chains, such as (reduced) cysteine residues.[23]

Importantly, the resulting conjugate was found to be catalytically active in the MT assay, with 51.2% zinc released in 60 min by 1.25 μM conjugate (0.625 μM catalyst) (Table 2, Fig. 4). Although the conjugate's activity was lower than the one of unbound **3**, it was still well within the range of good antioxidant catalysts. In addition, this is the first time a protein-bound catalyst at nanomolar catalyst concentration has shown activity in the MT assay. The reduced activity of the conjugate when compared to the unbound catalyst is not too surprising,

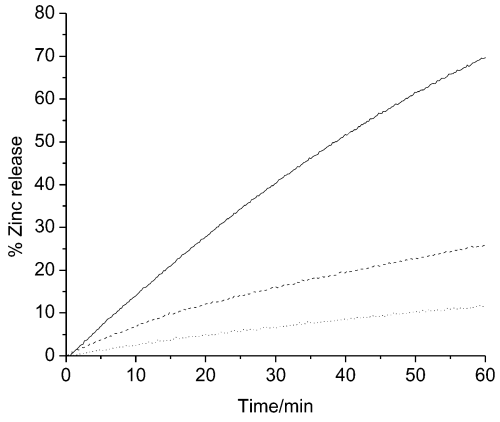

Fig. 4 Kinetic traces of the zinc release in the MT assay for the protein–catalyst conjugate in the presence of 500 μM ^{t}BuOOH (solid line, initial rate 7.95×10^{-10} M s^{-1}). Controls: the assay with protein–catalyst conjugate but without ^{t}BuOOH (broken line, initial rate 3.59×10^{-10} M s^{-1}), and the assay with ^{t}BuOOH but without protein–catalyst conjugate (dotted line, initial rate 1.28×10^{-10} M s^{-1}). These traces were recorded by continuous monitoring at 25 °C. Baseline corrected with 5% experimental error. Initial rates were calculated for 2–12 min.

and steric hindrance, as well as the chemical changes at the NH_2 group might play a role. It should be noted that HSA is not soluble in methanol and the PhSH-based assay described above could therefore not be used here for this conjugate.

Considered together, the results presented as part of this initial study show that it is feasible to design, synthesise and select effective redox catalysts that can be attached to proteins. From a pharmacological perspective, such constructs are likely to become increasingly important in the future, since they provide an elegant way to combine drug activity with selected transport properties, exploiting the natural transport pathways of the human body by using natural proteins as vehicles.

Although future pharmacological studies on these conjugates are all important, chemical techniques such as Cyclic Voltammetry and biochemical assays play a major role in designing and selecting suitable agents. CV, for example, provides valuable insights into the redox activity of such compounds, and might even serve as predictor of *in vitro* activity.[14,20] On the other hand, biochemical assays such as the GPx assay and the MT oxidation assay, as well as cell culture, enable the selection of suitable candidate compounds to be transported.

The next steps of this study will, of course, have to involve screening the conjugate in other *in vitro* assays, to look at the effects of the conjugate on cells and whole organisms, and to see if the transport properties of HSA are still intact. In addition, possible immunogenic aspects of a conjugate must always be considered, even if it is based on a human protein. Previous studies, using reduced and NO-modified HSA, have been successful in this respect, and this also bodes well for a catalyst–protein conjugate.[9]

The results confirm that it is possible to load chemically simple, yet catalytically highly active enzyme mimics onto HSA. The pharmacokinetic properties of the resulting conjugate(s) are currently being evaluated, with the aim to shuttle catalytic drugs along well-defined physiological distribution pathways of specific proteins. In the long term, this might provide the basis for novel antioxidant drug conjugates that can be organ site-directed by exploiting the transport properties of selected human proteins, some of which exhibit transport proteins considerably more interesting than the ones of HSA.

Experimental

Materials

All chemicals, reagents and media were obtained from Sigma-Aldrich (Poole, UK) unless stated otherwise. Cd,Zn-metallothionein was purchased from Sigma, and Zn_7–MT was reconstituted and purified according to a standard method.[24] SK-MES-1, human Caucasian lung squamous carcinoma, cell line was purchased from European Collection of Animal Cell Cultures (ECAC) 93120837. This cell line was chosen because it is known to have a high level of intracellular OS. Penicillin/streptomycin, heat inactivated foetal calf serum and L-glutamine were from BioWhittaker (Berkshire, UK) and poly-D-lysine coated 96 well plates from Greiner Bio-One Ltd (Gloucestershire, UK). Solutions and buffers were prepared in Milli Q water and nitrogen flushed prior to use. UV/VIS spectra were recorded on a CARY 50 *Bio* spectrophotometer (Varian). Cyclic Voltammetry (CV) was performed on a 100B/W workstation (BAS).

Synthesis of organotellurides 1–4

1 was synthesised according to literature, and analytical data obtained for the compound (melting point, elemental analysis, ^{1}H NMR and ^{13}C NMR) were in agreement with published results.[15] The same synthetic approach, with different starting materials and purification methods, was then employed to synthesise **2** to **4**.

2 was synthesised from bis(4-aminophenyl) ditelluride and 4-iodoanisole, with bis(4-aminophenyl) ditelluride synthesised according to literature.[25,26] **2** was purified by column chromatography (Silica) using hexane–$CHCl_3$ (4 : 6) as the eluent (R_f = 0.17), and obtained as brown crystals (9% yield). Melting point: 77.8 °C. Elemental analysis (%) found: C 47.58 H 3.78 N 4.02 (Calc. C 47.46, H 3.95, N 4.20). MS (El) *m/z* (%): 329.93 (100), 327.93 (90), 325.92 (60), 324.92 (25). ^{1}H NMR (300 MHz, $CDCl_3$): δ, 7.52–7.59 (dd, 4H), 6.73–6.76 (d, 2H), 6.54–6.56 (d, 2H), 3.7 (s, 3H), 3.6 (br, 2H). ^{13}C NMR (400 MHz, $CDCl_3$): δ, 146.57, 115.28, 138.94, 140.43, 116.34, 55.12, 159.43, 104.91, 100.82.

3 was synthesised from bis(2-aminophenyl) ditelluride and 4-iodoanisole, with bis(2-aminophenyl) ditelluride synthesised according to literature.[26] **3** was purified by column chromatography (Silica) using hexane–$CHCl_3$ (2 : 3) as the eluent (R_f = 0.48). It was obtained as a tan powder in 46% yield. Melting point: 56.3 °C. Elemental analysis (%) found: C 47.46, H 3.91, N 3.96 (Calc C 47.77, H 4.01, N 4.29). MS (El) *m/z* (%): 329.0 (100), 328 (35), 327.0 (99), 325.0 (85). ^{1}H NMR (300 MHz, $CDCl_3$): δ, 7.8 (dd, 1H), 7.50–7.60 (dd, 2H), 7.20 (m, H), 6.90 (dd, 2H), 6.75–6.80 (dd, 2H), 6.66 (ddd, 2H), 4.16 (br, 2H), 3.67 (s, 3H). ^{13}C NMR (400 MHz, $CDCl_3$): δ, 149.55, 114.1, 130.9, 119.3, 141.96, 138.36, 115.56, 55.2, 159.6, 101.2, 103.0.

4 was synthesised from bis(2-aminophenyl) diselenide and 4-iodoanisole. The resulting oil was purified by column chromatography (basic Alumina) using $CHCl_3$–hexane (1:9) as the eluent (R_f = 0.37). The product was obtained as red crystals in 49% yield. Melting point: 61.2 °C. Elemental analysis (%) found: C 55.7, H 4.61, N 4.93, (Calc C 56.12, H 4.71, N 5.03). MS (El) *m/z* (%): 281.1 (100), 279 (40). ^{1}H NMR (300 MHz, $CDCl_3$): δ, 7.6 (dd, 1H), 7.34 (dd, 2H), 7.24 (m, 1H), 6.86 (dd, 2H), 6.80 (dd, 2H), 6.76–6.71 (dd, 2H), 4.26 (br, 2H), 3.75 (s, 3H). ^{13}C NMR (400 MHz, $CDCl_3$): δ, 148.0, 118.8, 130.5, 121.2, 137.5, 132.2, 115.1, 55.3, 158.8, 121.2, 114.4.

Cyclic voltammetry

Cyclic voltammograms were recorded in nitrogen purged, 50 mM potassium phosphate buffer (pH 7.4), containing 30% methanol, using a 3 mm glassy carbon working electrode, a standard Ag/AgCl reference electrode (SSE) and a platinum wire counter electrode, with a potential range of 0 mV to +1200 mV *vs.* SSE, at scan rates of 100 to 500 mV s^{-1}.[20] The pH study was performed in 50 mM potassium phosphate buffer, containing 30% methanol, and a pH between 3 and 9.

In vitro assays

The thiophenol (PhSH) assay indicative of GPx-like catalytic activity was performed according to an established literature method.[16] In short, 100 μM of compound was added to a 1 mM methanolic solution of PhSH, the reaction initiated by addition of 2 mM H_2O_2 and monitored at 305 nm for 15 min at 25 °C. Initial rates were obtained for the interval of 1 and 2 min.

Since this assay is performed in methanol, it avoids solubility problems often associated with organochalcogens in water. On the other hand, the assay is unsuitable for proteins such as HSA.

The assay was therefore complemented by the metallothionein (MT) oxidation assay.[14,17] MT is a small zinc–sulfur protein that binds seven zinc ions in a Zn_4Cys_{11} and Zn_3Cys_9 cluster. Oxidative zinc release from MT is slow in the presence of most oxidants, and enhancement of zinc release, *e.g.* by catalysts, can easily be monitored spectrophotometrically using a chromophoric dye.[14] Unlike other, enzyme based peroxidation assays, the MT is very robust, highly reproducible and relatively simple to use with a wide range of compounds, proteins and oxidants.

Briefly, 0.5 μM MT were incubated at 25 °C with catalyst (100 or 200 nM) and 500 μM *tert*-butyl hydroperoxide in 20 mM HEPES, pH 7.4 containing 100 μM of the chromophoric zinc chelator 4-(2-pyridylazo)resorcinol (PAR). The reaction was monitored continuously at 500 nm for 60 min and initial rates were recorded for 2 to 12 min.

Cell culture

SK-MES-1 cells were grown in minimal essential medium supplemented with 10% (v/v) foetal calf serum (FCS), 1% (v/v) non-essential amino acids, 2 mM L-glutamine, penicillin (100 units ml^{-1}) and streptomycin (100 μg ml^{-1}) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Cells were routinely harvested by trysinisation when cells approached subconfluency. In growth media, cells were seeded (4000 per well) in 96 well poly-D-lysine plates and incubated overnight. Then, an assessment of compounds for their impact on cell survival rates were made, using the MTT assay[27] in which the FCS was replaced with 0.2% (w/v) bovine serum albumin. Compounds (1 nM–10 μM) were incubated for 23 h with cells pre-exposed to either 0 or 50 μM H_2O_2 (3 h). MTT solution was incubated (final concentration 0.5 mg ml^{-1}) in media with cells for 3 h, and the ensuing formazan crystals formed were dissolved in DMSO and quantified spectrophotometrically at 540 nm.

Synthesis and purification of the HSA–catalyst conjugate

Using the thiophosgene coupling method,[13] **3** (2 mg) was incubated with a 200-fold excess of thiophosgene in acetone for 1 h at room temperature. Solvent and excess thiophosgene were removed under vacuum. The resulting compound was dissolved in DMSO and added to HSA (10 mg ml^{-1} in carbonate buffer, pH 9.5) as 10-fold molar excess. After 3 h with continuous mixing at room temperature, the mixture was purified twice by gel filtration chromatography (PD-10 column followed by a Sephadex G-50 column, 25 cm long × 1.8 cm diameter, with 20 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (Na^+-HEPES, pH 7.4)). In deviation from the literature method using just a PD-10 column, this double purification method was chosen to avoid carrying over any unbound label. Labelled HSA was characterised by UV/VIS spectrometry, and protein content was determined using the Bradford assay.

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